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Utilisation of 5′ substituted Nucleosides for Resistance Formation in Cytostatic Treatment, and Drug containing these Nucleosides.

The occurrence of "drug resistance" is the main reason for failure in cancer chemotherapy. Tumours which initially react sensitively to cytostatic agents very frequently recover after a certain treatment time and theN are resistant to the effectS of various types of antineoplastic drugs. Although the problem of "drug resistance" has been known since 1948, the beginning of cancer therapy with cytostatic agents, until now no way has been found of preventing the formation of resistance. A characteristic of all highly resistant tumours and cell strains investigated to date is the amplification (multiplication) of a small group of genes. As a result of this DNA or gene amplification, an increased expression of these genes can be shown, which leads to resistance to the drug. As a result of this DNA amplification an increased expression of various genes can be proven. Protective proteins which serve to shuttle toxins out of the cell, can thus be formed in increased quantities (P-glycoprotein). This effect is also known as "multi-drug resistance" (MDR).

In addition to MDR, the degree of amplification of certain genes, above all certain oncogenes, correlates with the degree of malignancy. Thus the chances of survival with an amplification of ERVV2, RASKI, INT3, HST1, MYC and KSRAM in stomach cancer (Hirohasi, S., and Sugimura, T. Genetic alterations in human gastric cancer. Cancer cells (Cold Spring Harbor), 3:49-52, 1991) and ERBB2 und MYC in ovarian carcinoma (Sasano, H., Garrett, C.T., Wilkinson, D.S., Silverberg, S., Comerford, J., and Hyde, J. Protooncogene amplification and tumor ploidy in human ovarian neoplasm. Hum.Pathol., 21:382-391,1990), are very poor. In breast cancer the amplification of MYC correlates (Borg, A., Baldetorp, B., Fernö, M., Olsson, H., and Sigurdsson, H. C-myc amplification is an independent prognostic factor in postmenopausal breast cancer. Int. J. Cancer, 51:687-691,1992) and coamplification of INT2 and HST1 (Borg, A., Sigurdsson, H., Clark, G..M., et al., Association of INT2/HST1 coamplification in primary breast cancer with hormone-dependent phenotype and poor prognosis. Br.J.Cancer, 63:136142, 1991) with the progress of the disease. The amplification of ERBB2 (Descotes, F., Pavy, J.-J., and Adessi, G.L. Human breast cancer: Correlation study between HER-2/neu amplification and prognostic factors in an unselected population. Anticancer Res., 13:119-124,1993) und EGFR (Klijn, J.G.M., Berns,P.M.J.J.,

Schmitz, P.I.M., and Foekens, J.A. The clinical significance of epidermal growth factor receptor (EGF-R)in human breast cancer: a review on 5232 patients. Endocr.Rev., 13:3-17,1992)is linked to a poor prognosis. In oesophageal cancer the coamplification of HST1 and INT2 correlates with the degree of metastasis (Experiment,T., Tahara, E., Kajiyama, G., Sakamoto, H.,Terada,M., and Sugimura, T. High incidence of coamplification of hst-1 and int-2 genes in human esophageal carcinomas. Cancer Res. 49:5505-5508,1989).In summary it can be ascertained that by means of chronic treatment with carcinogenic cytostatic agents, the induced gene amplification leads not only to resistance to this treatment, but also to the over-expression of certain oncogenes which control the degree of malignancy.

A series of substances have been described which counteract the acquired drug resistance. Among these are the work described by Kennedy (Kennedy, A.R., Prevention of Carcinogenesis byProtease Inhibitors, Cancer Res., 54:1999-2005,1994) on the anti-carcinogenic effects of protease inhibitors. These can suppress carcinogen-induced gene amplification to almost normal levels. Kennedy observed that radiation-induced gene amplification is suppressed in the same way, as

corresponds with its capacity to suppress radiation-induced transformation, so that a relationship between these two processes can be assumed. In addition, protease-inhibitors act as antagonists of (co-recombinogenic) tumour inducers during the initiation of transformation in vitro. Protease-inhibitors are also described as effective anti-promoters in in vivo experiments (Troll, W., Klassen, A., and Janoff, A. Tumorigenesis in mouse skin: inhibition by synthetic inhibitors of proteases. Science (Washington DC) 169:1211-1213, 1970).

It is known from the source Moscow, I.A., and Cowan, K.H. Multidrug resistance. J.Natl.Cancer Inst. 80: 14-20, 1988,that verapamil acts against MDR. This "calcium channel blocker" increases the cycotoxicity by increasing the intracellular accumulation of drugs. This probably occurs due to an effect on the P-glycoprotein or other transport proteins. The toxicity of these and similar substances such for example as quinidine, opposes their clinical use.

Proceeding from this point it is the object of the present invention to propose an effective substance for preventing or reducing resistance formation against treatment with cytostatic agents, and to propose a corresponding drug.

The object as regards the substance is achieved by the characterising features of claim 1, and with respect to the drug by the features of claim 9.

Thus it is proposed according to the invention to prevent or reduce resistance formation by simultaneous administration of 5′ substituted nucleosides and a cytostatic agent. It has become surprisingly apparent that 5′ nucleosides prevent or at least attenuate the occurrence of carcinogen-induced gene amplifications. This offers the possibility of preventing the occurrence of resistances against this drug and also of influencing the degree of malignancy by means of simultaneous administration of these nucleosides with a cytostatic agent.

The following are examples of 5′ nucleosides: 5-(2-bromovinyl-2′-deoxyuridine (BVDU), (E)-5-(2-bromovinyl)-1-ß-D-arabinofuranosyluracil, (E)-5-(2-bromovinyl-2′-deoxy-41-thiouridine. Fig. 2: 5-iodo-2′-dexycytidin, 5-iodo-2′-deoxyuridine, 2′-Deoxy-5-trifluoromethyluridin,particularly preferred is BVDU and (E)-5-(2-Bromovinyl-)uracyl (BVU).

The invention further relates to drugs for preventing resistance formation against cytostatic

treatment, said drugs containing 5′ nucleosides. The 5′ nucleosides are contained in the formulation of the drug in a quantity from which there results a concentration of 0.02 µg/ml to 10 µg/ml in the blood. It was shown in experiments that the optimal range lies at 0.05 µg/ml to 5 µg/ml.

The cytostatic agents can be contained in the formulation in the quantities previously normal (Oshiro, Y., Piper, C.E., Balwierz, P.S., and Garriot, M.L. (1992) Genotoxic properties of (E)-5(2-bromovinyl)-21-deoxyuridine (BVDU). Fundamental and Applied Toxicology, 18, 491-498). Examples of cytostatic agents are cyclophosphamide and other alkylating agents, anti-metabolites such as methotrexate, alkaloids such as vinblastin, antibiotics such as bleomycin, cisplatin and other materials. Other examples of cytostatic agents can be seen in "Red List 1995", Editio Cantor Verlag für Medizin und Naturwissenschaften, Aulendorf/Württ., 1995.

The carrier, additive and auxiliary materials correspond to those previously known from prior art. The drug itself can be present in a solid or a liquid form, or also as a spray.

The invention will be explained in more detail in the following with reference to model experiments.

A. Model substances

There is used for investigation of amplification phenomena a hamster cell strain which contains a virus (simian virus 40) integrated in the genome. It is known for this cell strain that an addition of genotoxic substances, but also various "non-genotoxic" carcinogens and tumour inducers leads to amplification of SV40-DNA in the hamster genome. The method is based on the fact that marked SV40-DNA with hamster cell-DNA containing SV40-DNA in an amplified number, serving as a probe, is hybridised. The quantity of bound probe gives an insight into the degree of amplification of the integrated virus-DNA.

In order to determine the degree of amplification the albumen-gene-DNA is measured simultaneously with the SV40-DNA. For the albumen-gene, contrary to SV40-DNA, is not amplified in the cell. Accordingly the value of the relative SV40-DNA content results from the quotient of the signal of the DNA probe hybridised with SV40-DNA to the signal of the DNA probe hybridised with albumen gene

from the same SV40 transformed embryonic CO631 hamster cells.

The following served as model substances:

1. Mutagens und recombinogenic genotoxic carcinogens (positive control)

   Triethylenmelamin (TEM) 4-Nitrochinolin 1-oxide (4-NQO)

2. Non-carcinogens (negative-control), which induce neither mutations nor recombinations

   acetone

   dimethylformamide

3. Co-recombinogenic Tumour-Inducers

   mezerein

   12-0-tetradecanoyl-phorbol-13-acetate (TPA)

   chrysarobin

   coumarin

4. Recombinogenic non-genotoxic carcinogens with unknown effective mechanism

   thioacetamide

   acetamide

5. Experiments after metablisation by rat liver microsomes (S9-Mix) and without S9-Mix

experiments acts

with S9-Mix anti-recombinogen und
without S9-Mix co-recombinogen

The effect of the abovenamed model substances alone or in combination with a carcinogen was tested in the gene amplification system.

The results with the model substances are shown in Figures 1 to 3.

The non-carcinogens acetone and dimethylformamide reveal no effect.

All other substances, the non-genotoxic carcinogens with unknown effective mechanism, thiocetamide and acetamide, the genotoxic carcinogens TEM and 4-NQO and the tumour inducers mezerein, 12-0-tetradecanoyl-phorbol-13-acetate (TPA), chyrsarobin and coumarin, given alone, increase the SV40 gene amplification.

Experiments with S9-mix reduces the amplifying effect of methotrexate (MTX), and without S9-mix it increases the amplifying effect of amino-6-mercaptopurin (AMP).

These results show an agreement between the initiation of recombination and SV40 gene amplification.

B. Inhibition of carcinogen-induced gene amplification by (E)-5-(2-Bromovinyl-)-2'-deoxyuridinee (BVDU)

The results are assembled in Figures 4 to 7.

In experiments with yeasts (Figure 4), BVDU had revealed an anti-recombinogenic and co-mutagenic effect. This effect was more visible in the presence of liver michrosomes (S9-mix) in lower concentration than in the absence of S9-mix, and was also much more defined. Thus metabolisation of BVDU reinforces the anti-recombinogenic effect.

BDVU reveals in clinically relevant doses an inhibition of AMP-induced gene amplification. The effect starts up at about 0.05 µg/ml and, dependent on dose, leads at 5 µg/ml to total inhibition of the AMP-induced gene amplification (Figure 5).

The independent repeat experiment confirms the result (Figure 6). In addition, BVDU alone appears slightly to lessen the spontaneous degree of amplification.

The addition of S9-mix likewise reveals a lowering of AMP-induced gene amplification. This however occurs in a lower dose range than in the experiments without S9-mix. Possible metabolisation of BVDU thus appears further to reinforce the amplification-inhibiting effect (Figure 7). This would further underline the relevance of the results.

In summary it can be stated that BVDU inhibits carcinogen-induced gene amplification. This opens up the possibility, with simultaneous administration of BVDU with a cytostatic agent, of preventing the occurrence of resistances to this drug and of lowering the malignancy.

C. Prevention of the Formation of "multi-drug resistance" (MDR) in Human and Animal Tumour Cells to Treatment with Cytostatic Agents by simultaneous Administration of BVDU.

The human tumour cell strain K562-WT (Figure 8) and the tumour cell strain F46-WT of the mouse (Figure 9) (WT = wild type = sensitive to cytostatic treatment = no amplification of the MDR-gene) was treated over several weeks with staged increase in concentrations of

adriamycin. During the treatment the cells acquired a resistance to this treatment. Whereas with non-resistant cells, 20 ng/ml adriamycin at a treatment time of 4 days has a severely toxic effect, the cells after long term treatment with staged increase in concentration became totally insensitive to 20 ng/ml adriamycin (Figures 8 and 9). The formation of resistance is based on the amplification of the MDR gene. This is indicated with the aid of the Northern Technique, a method for indicating RNA molecules, i.e. the transcription of a gene, using the MDR gene as a probe (Figure 10). Resistant cells show a band, non-resistant cells (condition at the start of treatment) have no band.

In parallel experiments with adriamycin with either 0.5 or 1 µg/ml BVDU given together (BVDU acts in human tumour cells only from about 10 µg/ml in a toxic manner, and in mouse cells from about 8 µg/ml (Figures 8 and 9)). BVDU prevents the formation of resistance to adriamycin. The tumour cells remain sensitive to the cytostatic treatment and die off. The effect of BVDU is so intense that the treatment must be interrupted by rest stages (growth without substances), so that the experiment extends over 6 to 8 weeks.

BVDU + adriamycin treatment leads to a considerably weaker amplification of the MDR gene than adriamycin treatment alone (Figure 10). The effect of the BVDU treatment is in reality a much greater than is expressed by the attenuation of the band. At the end of the treatment, namely, there remain only cells which have acquired at least a certain resistance to the adriamycin treatment. The cells which have remained non-resistant as a result of the BVDU treatment have already previously died off. The actual relevant effect, and not detectable with the aid of the Northern Technique, therefore consists in die-off of the non-resistant cells, which is measured in the inactivation curves (Figures 8 and 9).

As the formation of resistance to cytostatic treatment in human tumours is likewise based on the amplification of the MDR gene, the combination of BVDU with an optional cytostatic agent offers the possibility of carrying out therapy at low doses and over longer periods of time than previously. Moreover, the prevention of the formation of resistance is of great importance also for other applications.

D. Prevention of the formation of "multi-drug-resistance" (MDR) in tumour cells to cytostatic treatment by simultaneous administration of anti-recombinogenic 5' substituted nucleosides

It can be seen in Figures 11 and 12 that the anti-recombinogenic effect is not specific only to BVDU, but is a property of all 5′ substituted nucleosides.

Fig. 11 thus shows the anti-recombinogenic effects of (E)-5-(2-bromovinyl)-21-deoxyuridine, (E)-5-(2bromovinyl)-1-ß-D-arabinofuranosyl-uracil and(E)-5(2-bromovinyl)-21-deoxy-41-thiouridine in Saccharomyces cerevisiae MP1, and

Fig. 12 the anti-recombinogenic effects of 5-iodo2′-deoxycytidin, 5-iodo-2′-deoxyuridine and 2′-deoxy-5-trifluoromethyluridin in saccharomyces cerevisiae MP1.

The tumour cell strain F4-6-WT of the mouse (WT = wild type = sensitive to cytostatic treatment = no amplification of the MDR gene) was treated over several weeks with staged increases in concentration of adriamycin. During the treatment the cells acquired a resistance to this treatment. Whereas 20 ng/ml of adriamycin at a treatment time of 4 days has an extremely toxic effect on non-resistantcells, the cells after a long term treatment with staged increases in concentration had become totally insensitive to 20 ng/ml adriamycin (Figures 13 and 14). The formation of

resistance is based on the amplification of the MDR gene. This was indicated with the aid of the Northern Technique, a method for indicating RNA molecules, i.e. the transcription of a gene, using the MDR gene as a probe (Figure 15). Resistant cells show a band, non-resistant cells (condition at the start of treatment) show no band. The levels of β-actin mRNA were likewise analysed as comparison. β-actin was used as an internal control for the RNA quantity.

In parallel experiments, adriamycin was administered with 1 µg/ml of a respective 5′ substituted nucleoside. All six 5′ substituted nucleosides prevent the formation of resistance to adriamycin. The tumour cells remain sensitive to the cytostatic treatment and die off. The effect of the 5′ substituted nucleosides was so intense that the treatment had to be interrupted by rest phases (growth without substances), so that the experiment extended over 6 to 8 weeks.

Figure 15 shows the Northern Blot Analysis of RNA:

Expression of the MDR genes in the tumour cell strain F4-6-WT of the mouse. The levels of β-actin mRNA were likewise also analysed for comparison. β-actin was used as an internal control for the RNA quantity.

Pos. adriamycin-resistant F4-6-WT cells

neg. adriamycin-sensitive F4-6-WT cells

1 1 µg/ml(E)-5-(2-bromovinyl)-2′-deoxyuridine + adriamycin

2 1 µg/ml (E)-5-(2-bromovinyl-1-ß-D-arabinofuranosyl-uracil + adriamycin

3 1 µg/ml(e)-5-(2-bromovinyl-2′-deoxy-4′-thiouridine + adriamycin

4 1 µg/ml 5-iodo-2′-deoxycytidin + adriamycin

5 1 µg/ml 5-iodo-2′-deoxyuridine + adriamycin

6 1 mg/ml 2′-Deoxy-5-trifluoromethyluridine + adriamycin

7 1 µg/ml (E)-5-(2-bromovinyl-)-2′-deoxyuridine (BVDU) + adriamycin

5′ substituted nucleoside + adriamycin treatment leads to a considerably weaker amplification of the MDR gene than adriamycin treatment alone (Figure 15). The effect of the treatment is in reality much more intense than is expressed by the attenuation of the bands. At the end of the treatment, namely, only cells remain which have acquired at least a certain resistance to the adriamycin treatment. The cells which have remained non-resistant

due to the BVDU treatment have already previously died off. The actually relevant effect, and not detectable with the aid of the Northern Technique, therefore consists in the die-off of the non-resistant cells, which was measured in the inactivation curves (Figures 13 and 14).